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(54) Title: OXIDIZING ENZYMES IN THE MANUFACTURE OF PAPER MATERIALS

(57) Abstract: The use of fatty acid oxidizing enzymes in the manufacture of paper materials, such as paper, linerboard, corrugated paperboard, tissue, towels, corrugated containers and boxes. Examples of fatty acid oxidizing enzymes are oxygenases classified as EC 1.13.11, including any of the sub-classes thereof, such as lipoxygenase, EC 1.13.11.12. The effect of these enzymes is that the deposition of pitch is reduced, and bleaching and de-inking effects are also observed on the paper pulp and the resulting paper material. The fatty acid oxidizing enzyme can be used in combination with a substrate, with proteases, liguses, xylanases, cutinases, oxidoreductases, cellulases, endoglucanases amylases, mannanases, steryl esterases, and/or cholesterol esterases; or with surfactants and other adjuvants.

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OXIDIZING ENZYMES IN THE MANUFACTURE OF PAPER MATERIALS

TECHNICAL FIELD

The present invention relates to the use of a fatty acid oxidizing enzyme in the manufacture of a paper material, as well as a process for the manufacture of a paper material, the process comprising a step in which papermaking pulp and/or papermaking process water is treated with a fatty acid oxidizing enzyme.

BACKGROUND ART

It is well-known to use enzymes in the manufacture of paper materials. Examples of enzymes used for this purpose are proteases, lipases, xylanases, amylases, cellulases, as well as various oxidizing enzymes such as oxidoreductases (phenol oxidizing enzymes), for example laccases and peroxidases.

The effects of these enzymes are wide-spread, e.g. control of various deposits such as pitch, strength-improvement, de-inking, drainage improvement, tissue softening, bleaching etc.

In a papermaking process, dissolved and colloidal substances (DCS) are dispersed into the process water during the pulp and paper production. The DCS are often referred to as wood pitch or wood resin. Pitch causes problems in paper machines by sticking to the rollers and causing spots or holes in the paper material.

Wood contains about 1 to 10% of pitch or extractives in addition to its main components cellulose, hemicellulose and lignin. Major components of pitch are fatty acids, triglycerides, sterols, steryl esters and so-called resin acids, e.g. abietic acid.

WO 00/53843 discloses certain steryl esterase enzyme preparations and their use in the manufacture of paper to hydrolyze the steryl ester part of pitch.

US Patent No. 6,066,486 discloses an enzyme preparation comprising a cholesterol esterase derived from *Pseudomonas fragi*, and the use thereof to hydrolyze pulp resin.

JP 2000080581 discloses the use of certain peroxidases for the decomposition of abietic acid during pulping or paper making processes.

X. Zhang; Pulp & Paper Canada, 101:3 (2000), page 59-62, discloses studies of the ability of e.g. laccase to remove dissolved and colloidal substances.

Also Karlsson et al.: Reactivity of *Trametes* laccases with fatty and resin acids; Appl. Microbiol. Biotechnol. (2001) 55:317-320 discloses experiments in which laccases were used to treat a model pitch preparation.

However, none of the references cited above disclose the use of a fatty acid oxidizing enzyme as defined herein for the manufacture of a paper material.

SUMMARY OF THE INVENTION

The present inventors surprisingly found that certain oxidizing enzymes, viz. fatty acid oxidizing enzymes, are advantageous in the manufacture of paper materials. An important effect of these enzymes is that the deposition of pitch is reduced. Furthermore, these enzymes have a bleaching effect on the paper pulp and the resulting paper material. And finally, a de-inking effect has been observed, too.

DETAILED DESCRIPTION OF THE INVENTION

Paper and Pulp

By the term a "paper-making process" is meant a process, wherein the pulp is suspended in water, mixed with various additives and then passed to equipment in which the paper, cardboard, tissue, towel etc. is formed, pressed and dried.

The term "paper material" refers to products, which can be made out of pulp, such as paper, linerboard, corrugated paperboard, tissue, towels, corrugated containers or boxes.

The term "a papermaking pulp" or "pulp" means any pulp which can be used for the production of a paper material. For example, the pulp can be supplied as a virgin pulp, or can be derived from a recycled source. The papermaking pulp may be a wood pulp, a non-wood pulp or a pulp made from waste paper. A wood pulp may be made from softwood such as pine, redwood, fir, spruce, cedar and hemlock or from hardwood such as maple, alder, birch, hickory, beech, aspen, acacia and eucalyptus. A non-wood pulp may be made, e.g., from bagasse, bamboo, cotton or kenaf. A waste paper pulp may be made by re-pulping waste paper such as newspaper, mixed office waste, computer print-out, white ledger, magazines, milk cartons, paper cups etc.

In a particular embodiment, the papermaking pulp to be treated comprises both hardwood pulp and softwood pulp.

The wood pulp to be treated may be mechanical pulp (such as ground wood pulp, GP), chemical pulp (such as Kraft pulp or sulfite pulp), semichemical pulp (SCP), thermomechanical pulp (TMP), chemithermomechanical pulp (CTMP), or bleached chemithermomechanical pulp (BCTMP).

Mechanical pulp is manufactured by the grinding and refining methods, wherein the raw material is subjected to periodical pressure impulses. TMP is thermomechanical pulp, GW is groundwood pulp, PGW is pressurized groundwood pulp, RMP is refiner mechanical pulp, PRMP is pressurized refiner mechanical pulp and CTMP is chemithermimechanical pulp.

Chemical pulp is manufactured by alkaline cooking whereby most of the lignin and hemicellulose components are removed. In Kraft pulping or sulphate cooking sodium sulphide or sodium hydroxide are used as principal cooking chemicals. In these types of pulp, as a result of the alkaline cooking, the triglyceride part of pitch will be hydrolysed into fatty acids and

glycerol. Fatty acid oxidizing enzymes are particularly useful in the treatment of such pulps, because, as the designation tells, these enzymes will catalyze the further degradation of the fatty acids resulting from the alkaline hydrolysis of the triglycerides.

The Kraft pulp to be treated may be a bleached Kraft pulp, which may consist of softwood bleached Kraft (SWBK, also called NBKP (Nadel Holz Bleached Kraft Pulp)), hardwood bleached Kraft (HWBK, also called LBKP (Laub Holz Bleached Kraft Pulp and)) or a mixture of these.

The pulp to be used in the process of the invention is a suspension of mechanical or chemical pulp or a combination thereof. For example, the pulp to be used in the process of the invention may comprise 0%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80%, 80-90%, or 90-100% of chemical pulp. In a particular embodiment, a chemical pulp forms part of the pulp being used for manufacturing the paper material. In the present context, the expression "forms part of" means that in the pulp to be used in the process of the invention, the percentage of chemical pulp lies within the range of 1-99%. In particular embodiments, the percentage of chemical pulp lies within the range of 2-98%, 3-97%, 4-96%, 5-95%, 6-94%, 7-93%, 8-92%, 9-91%, 10-90%, 15-85%, 20-80%, 25-75%, 30-70%, 40-60%, or 45-55%.

In a particular embodiment of the use and the process of the invention, the chemical pulp is a Kraft pulp, a sulfite pulp, a semichemical pulp (SCP), a thermomechanical pulp (TMP), a chemithermomechanical pulp (CTMP), a bleached chemithermomechanical pulp (BCTMP). In particular embodiments the Kraft pulp is bleached Kraft pulp, for example softwood bleached Kraft (SWBK, also called NBKP (Nadel Holz Bleached Kraft Pulp)), hardwood bleached Kraft (HWBK, also called LBKP (Laub Holz Bleached Kraft Pulp and)) or a mixture thereof.

Process Conditions

The process of the invention is particularly applicable to the oxidation and hydrolysis of compounds constituting the pitch during a pulping or paper-making process, e.g. to avoid pitch troubles.

The process of the invention may be applied to any pitch-containing pulp, especially to pulps with a considerable content of linoleic acid or other unsaturated free fatty acids.

In the case of paper and pulp processing, the process according to the invention can be carried out at any pulp production stage. The enzyme can be added to any holding tank, e.g. to a pulp storing container (storage chest), storage tower, mixing chest or metering chest. The enzyme treatment can be performed before the bleaching of pulp, in connection with the pulp bleaching process or after the bleaching. When carried out in connection with pulp bleaching the enzyme preparation may be added together with bleaching chemicals such as chlorine, chlorine dioxide. Applying oxygen gas, hydrogen peroxide or ozone or combinations thereof may also carry out the bleaching of pulp. The enzyme preparation may also be added together with these substances. Preferably the enzyme preparation is added prior to bleaching. The enzyme can also be added to

the circulated process water (white water) originating from bleaching and process water (brown water) originating from the mechanical or chemimechanical pulping process. In a particular embodiment of a Kraft pulping process, the enzyme is added during the brown-stock washing.

In the present context, the term "process water" comprises i.a. 1) water added as a raw material to the paper manufacturing process; 2) intermediate water products resulting from any step of the process for manufacturing the paper material; as well as 3) waste water as an output or by-product of the process. In a particular embodiment, the process water is, has been, is being, or is intended for being circulated (re-circulated), i.e. re-used in another step of the process. The term "water" in turn means any aqueous medium, solution, suspension, e.g. ordinary tap water, and tap water in admixture with various additives and adjuvants commonly used in paper manufacturing processes. In a particular embodiment the process water has a low content of solid (dry) matter, e.g. below 20%, 18%, 16%, 14%, 12%, 10%, 8%, 7%, 6%, 5%, 4%, 3%, 20% or below 1% dry matter.

The use and process of the invention does not include the use of the lipoxygenase derived from *Magnaporthe salvinii* as described in Example 2 of PCT/DK02/00251 for bleaching dye for pulp industry in waste water.

The process of the invention may be carried out at conventional conditions in the paper and pulp processing. The process conditions will be a function of the enzyme(s) applied, the reaction time and the conditions given.

The enzyme of the invention should be added in an effective amount. By the term "effective amount" is meant the amount sufficient to achieve the desired and expected effect, such as oxidizing pitch components, obtaining a desired bleaching and/or de-inking etc.

In a particular embodiment, the dosage of the fatty acid oxidizing enzyme and additional enzymes, if any, is from about 0.1 mg enzyme protein to about 100.000 mg enzyme protein (of each enzyme) per ton of paper pulp.

In further particular embodiments, the amount of the fatty acid oxidizing enzyme and additional enzymes, if any, is in the range of 0.00001-20; or 0.0001-20 mg of enzyme (calculated as pure enzyme protein) per gram (dry weight) of lignocellulosic material, such as 0.0001-10 mg/g, 0.0001-1 mg/g, 0.001-1 mg/g, 0.001-0.1, or 0.01-0.1 mg of enzyme per gram of lignocellulosic material. Again, these amounts refer to the amount of each enzyme.

The enzymatic treatment can be done at conventional consistency, e.g. 0.5-10 % dry substance. In particular embodiments, the consistency is within the range of 0.5-45; 0.5-40; 0.5-35; 0.5-30; 0.5-25; 0.5-20; 0.5-15; 0.5-10; 0.5-8; 0.5-6; or 0.5-5% dry substance.

The enzymatic treatment may be carried out at a temperature of from about 10 to about 100°C. Further examples of temperature ranges (all "from about" and "to about") are the following: 20-100, 30-100, 35-100, 37-100, 40-100, 50-100, 60-100, 70-100, 10-90, 10-80, 10-70, 10-60, and 30-60°C, as well as any combination of the upper and lower values here indicated. A typical

temperature is from about 20 to 90°C, or 20 to 95°C, preferably from about 40 to 70°C, or 40 to 75°C.

The enzymatic treatment may be carried out at a pH of from about 2 to about 12. Further examples of pH ranges (all "from about" and "to about") are the following: 3-12, 4-12, 5-12, 6-12, 7-12, 8-12, 9-12, 2-11, 2-10, 2-9, 2-8, 4-10, 5-8 as well as any combination of the upper and lower values here indicated. A typical pH range is from about 2 to 11, preferably within the range from about 4 to 9.5, or 6 to 9.

A suitable duration of the enzymatic treatment may be in the range from a few seconds to several hours, e.g. from about 30 seconds to about 48 hours, or from about 1 minute to about 24 hours, or from about 1 minute to about 18 hours, or from about 1 minute to about 12 hours, or from about 1 minute to 5 hours, or from about 1 minute to about 2 hours, or from about 1 minute to about 1 hour, or from about 1 minute to about 30 minutes. A typical reaction time is from about 10 minutes to 3 hours, 10 minutes to 10 hours, preferably 15 minutes to 1 hour, or 15 minutes to 2 hours.

Molecular oxygen from the atmosphere will usually be present in sufficient quantity, if required. Therefore, the reaction may conveniently be carried out in an open reactor, i.e. at atmospheric pressure.

Various additives over and above the fatty acid oxidizing enzyme and additional enzymes, if any, can be used in the process or use of the invention. Surfactants and/or dispersants are often present in, and/or added to a papermaking pulp. Thus the process and use of the present invention may be carried out in the presence of an anionic, non-ionic, cationic and/or zwitterionic surfactant and/or dispersant conventionally used in a papermaking pulp. Examples of anionic surfactants are carboxylates, sulphates, sulphonates or phosphates of alkyl, substituted alkyl or aryl. Fatty acids are examples of alkyl-carboxylates. Examples of non-ionic surfactants are polyoxyethylene compounds, such as alcohol ethoxylates, propoxylates or mixed ethoxy-/propoxylates, poly-glycerols and other polyols, as well as certain block-copolymers. Examples of cationic surfactants are water-soluble cationic polymers, such as quartenary ammonium sulphates and certain amines, e.g. epichlorohydrin/dimethylamine polymers (EPI-DMA) and cross-linked solutions thereof, polydiallyl dimethyl ammonium chloride (DADMAC), DADMAC/Acrylamide co-polymers, and ionene polymers, such as those disclosed in US patents nos. 5,681,862; and 5,575,993. Examples of zwitterionic or amphoteric surfactants are betains, glycinates, amino propionates, imino propionates and various imidazolin-derivatives. Also the polymers disclosed in US patent no. 5,256,252 may be used.

Also according to the invention, surfactants such as the above, including any combination thereof may be used in a paper making process together with a fatty acid oxidizing enzyme as defined herein, and included in a composition together with such enzyme. The amount of each surfactant in such composition may amount to from about 8 to about 40% (w/w) of the

composition. In particular embodiments the amount of each surfactant is from about 10 to about 38, or from about 12 to about 36, or from about 14 to about 34, or from about 16 to about 34, or from about 18 to about 34, or from about 20 to about 34, or from about 22 to about 34, or from about 24 to about 34, or from about 26 to about 34, or from about 28 to about 32% (w/w).

In another particular embodiment, each of the above ranges refers to the total amount of surfactants.

Enzymes

EC-numbers may be used for classification of enzymes, e.g. lipase EC-number for enzymes having lipase activity, etc. Reference is made to the Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, Academic Press Inc., 1992.

It is to be understood that the term enzyme, as well as the various enzymes and enzyme classes mentioned herein, encompass wild-type enzymes, as well as any variant thereof that retains the activity in question. Such variants may be produced by recombinant techniques. The wild-type enzymes may also be produced by recombinant techniques, or by isolation and purification from the natural source.

In a particular embodiment the enzyme in question is well-defined, meaning that only one major enzyme component is present. This can be inferred e.g. by fractionation on an appropriate Size-exclusion column. Such well-defined, or purified, or highly purified, enzyme can be obtained as is known in the art and/or described in publications relating to the specific enzyme in question.

Fatty Acid Oxidizing Enzyme

The term "a" fatty acid oxidizing enzyme means at least one of such enzymes. The term "at least one" means one, two, three, four, five, six or even more of such enzymes.

In the present context, a fatty acid oxidizing enzyme is an enzyme which hydrolyzes the substrate linoleic acid more efficiently than the substrate syringaldazine. "More efficiently" means with a higher reaction rate. This can be tested using the method described in Example 2, and calculating the difference between (1) absorbancy increase per minute on the substrate linoleic acid (absorbancy at 234 nm), and (2) absorbancy increase per minute on the substrate syringaldazine (absorbancy at 530 nm), i.e. by calculating the Reaction Rate Difference (RRD) = $(d(A_{234})/dt - d(A_{530})/dt)$. If the RRD is above zero, the enzyme in question qualifies as a fatty acid oxidizing enzyme as defined herein. If the RRD is zero, or below zero the enzyme in question is not a fatty acid oxidizing enzyme.

In particular embodiments, the RRD is at least 0.05, 0.10, 0.15, 0.20, or at least 0.25 absorbancy units/minute.

In a particular embodiment of the method of Example 2, the enzymes are well-defined. Still further, for the method of Example 2 the enzyme dosage is adjusted so as to obtain a maximum absorbancy increase per minute at 234 nm, or at 530 nm. In particular embodiments, the maximum absorbancy increase is within the range of 0.05-0.50; 0.07-0.4; 0.08-0.3; 0.09-0.2; or 0.10-0.25 absorbancy units pr. min. The enzyme dosage may for example be in the range of 0.01-20; 0.05-15; or 0.10-10 mg enzyme protein per ml.

In the alternative, a "fatty acid oxidizing enzyme" may be defined as an enzyme capable of oxidizing unsaturated fatty acids more efficiently than syringaldazine. The activity of the enzyme could be compared in a standard oximeter setup as described in Example 1 of the present application at pH 6 and 30°C including either syringaldazine or linoleic acid as substrates.

In a particular embodiment, the fatty acid oxidizing enzyme is defined as an enzyme classified as EC 1.11.1.3, or as EC 1.13.11.-. EC 1.13.11.- means any of the sub-classes thereof, presently forty-nine: EC 1.13.11.1-EC 1.13.11.49. EC 1.11.1.3 is designated fatty acid peroxidase, and EC 1.13.11.- is designated oxygenases acting on single donors with incorporation of two atoms of oxygen.

In a further particular embodiment, the EC 1.13.11.- enzyme is classified as EC 1.13.11.12, EC 1.13.11.31, EC 1.13.11.33, EC 1.13.11.34, EC 1.13.11.40, EC 1.13.11.44 or EC 1.13.11.45, designated lipoxygenase, arachidonate 12-lipoxygenase, arachidonate 15-lipoxygenase, arachidonate 5-lipoxygenase, arachidonate 8-lipoxygenase, linoleate diol synthase, and linoleate 11-lipoxygenase, respectively).

In a further particular embodiment, the fatty acid oxidizing enzyme is a lipoxygenase (LOX), classified as EC 1.13.11.12, which is an enzyme that catalyzes the oxygenation of polyunsaturated fatty acids, especially *cis,cis*-1,4-dienes, e.g. linoleic acid and produces a hydroperoxide. But also other substrates may be oxidized, e.g. monounsaturated fatty acids.

Microbial lipoxygenases can be derived from, e.g., *Saccharomyces cerevisiae*, *Thermoactinomyces vulgaris*, *Fusarium oxysporum*, *Fusarium proliferatum*, *Thermomyces lanuginosus*, *Pyricularia oryzae*, and strains of *Geotrichum*. The preparation of a lipoxygenase derived from *Gaeumannomyces graminis* is described in Examples 3-4 of WO 02/20730. The expression in *Aspergillus oryzae* of a lipoxygenase derived from *Magnaporthe salvinii* is described in Example 2 of PCT/DK02/00251, and this enzyme can be purified using standard methods, e.g. as described in Example 4 of WO 02/20730.

Lipoxygenase (LOX) may also be extracted from plant seeds, such as soybean, pea, chickpea, and kidney bean. Alternatively, lipoxygenase may be obtained from mammalian cells, e.g. rabbit reticulocytes.

Lipoxygenase activity may be determined spectrophotometrically at 25°C by monitoring the formation of hydroperoxides. For the standard analysis, 10 micro liters enzyme was added

to a 1 ml quartz cuvette containing 980 micro liter 25 mM sodium phosphate buffer (pH 7.0) and 10 micro liter of substrate solution (10 mM linoleic acid dispersed with 0.2%(v/v) Tween20 (should not be kept for extended time periods)). The enzyme was typically diluted sufficiently to ensure a turn-over of maximally 10% of the added substrate within the first minute. The absorbance at 234 nm was followed and the rate was estimated from the linear part of the curve. The *cis-trans*-conjugated hydro(pero)xy fatty acids were assumed to have a molecular extinction coefficient of 23,000 $M^{-1}cm^{-1}$.

The fatty acid oxidizing enzyme may also be applied together with a substrate for the enzyme capable of enhancing the enzymatic effect. Suitable substrates are hydrolyzed oils such as oils from soybeans (rich in linoleic acid) or tall oil. Fatty acid substrates may be released from the added oil by lipolytic enzymes or produced during the Kraft pulping or sulphate cooking.

In particular embodiments the substrate is a compound with 1,4-pentadien structure, e.g. with *cis,cis*-1,4-pentadien structure, i.e. compounds having at least one such element in its structural formula. Examples of such substrates are unsaturated fatty acids, e.g. palmitoleic acid, oleic acid, linoleic acid, linolenic acid, and arachidonic acid, as well as their salts and esters, e.g. methyl- and ethyl-esters.

In further particular embodiments the substrate is linoleic acid; linoleic acid methyl or ethyl ester; linolenic acid, or linolenic acid methyl or ethyl ester.

To explore the effect of adding a substrate for the fatty acid oxidizing enzyme in question, the following method may be used: The spectrum of 10 mM abietic acid (emulsified in 0.2% Tween 20) is recorded. Characteristic peaks are observed around 200 nm and around 250 nm. In a first experiment, a fatty acid oxidizing enzyme is added to the abietic acid emulsion. In a second experiment, a substrate for the fatty acid oxidizing enzyme is also added. The enzyme is e.g. a lipoxygenase derived from *M. salvinii* as described above, and the substrate is e.g. linoleic acid. The degradation of abietic acid is followed spectrophotometrically, and the peaks around 200 nm and around 250 nm decrease more rapidly when linoleic acid is added together with the lipoxygenase.

In particular embodiments of the above method, and of the process of the invention, the substrate, e.g. linoleic acid, is added in an amount of 5-10000 ppm (mg/l), or 10-9000, 10-8000, 25-7500, 30-7000, 50-6000, 50-5000, 50-4000, 75-3000, 75-2500, 80-2000, 90-1500, 100-1000, 150-800, or 200-700 ppm. In Example 4, 333 ppm of linoleic acid was used together with a fatty acid oxidizing enzyme.

In further particular embodiments of the above method, and of the process of the invention, the fatty acid oxidizing enzyme is used in an amount of 0.005-50 ppm (mg/l), or 0.01-40, 0.02-30, 0.03-25, 0.04-20, 0.05-15, 0.05-10, 0.05-5, 0.05-1, 0.05-0.8, 0.05-0.6, or 0.1-0.5 ppm. The amount of enzyme refers to mg of a well-defined enzyme preparation.

In the process of the invention, the fatty acid oxidizing enzyme may be applied alone or together with an additional enzyme. The term "an additional enzyme" means at least one additional enzyme, e.g. one, two, three, four, five, six, seven, eight, nine, ten or even more additional enzymes.

The term "applied together with" (or "used together with") means that the additional enzyme may be applied in the same, or in another step of the process of the invention. The other process step may be upstream or downstream in the paper manufacturing process, as compared to the step in which the papermaking pulp or process water is treated with a fatty acid oxidizing enzyme.

In particular embodiments the additional enzyme is an enzyme which has protease, lipase, xylanase, cutinase, oxidoreductase, cellulase, endoglucanase, amylase, mannanase, steryl esterase, and/or cholesterol esterase activity. Examples of oxidoreductase enzymes are enzymes with laccase, and/or peroxidase activity. In a preferred embodiment, the additional enzyme is lipase.

The term "a step" of a process means at least one step, and it could be one, two, three, four, five or even more process steps. In other words the fatty acid oxidizing enzyme of the invention may be applied in at least one process step, and the additional enzyme(s) may also be applied in at least one process step, which may be the same or a different process step as compared to the step where the fatty acid oxidizing enzyme is used.

The term "enzyme preparation" means a product containing at least one fatty acid oxidizing enzyme. The enzyme preparation may also comprise enzymes having other enzyme activities, preferably lipolytic enzymes or enzymes having oxidoreductase activity, most preferably lipolytic enzymes. In addition to the enzymatic activity such a preparation preferably contains at least one adjuvant. Examples of adjuvants, which are used in enzyme preparations for the paper and pulp industry are buffers, polymers, surfactants and stabilizing agents.

Additional enzymes

Any enzyme having protease, lipase, xylanase, cutinase, oxidoreductase, cellulase endoglucanase, amylase, mannanase, steryl esterase, and/or cholesterol esterase activity can be used as additional enzymes in the use and process of the invention. Below some non-limiting examples are listed of such additional enzymes. The enzymes written in capitals are commercial enzymes available from Novozymes A/S, Krogshoejvej 36, DK-2880 Bagsvaerd, Denmark. The activity of any of those additional enzymes can be analyzed using any method known in the art for the enzyme in question, including the methods mentioned in the references cited.

Examples of cutinases are those derived from *Humicola insolens* (US 5,827,719); from a strain of *Fusarium*, e.g. *F. roseum culmorum*, or particularly *F. solani pisi* (WO 90/09446; WO

94/14964, WO 94/03578). The cutinase may also be derived from a strain of *Rhizoctonia*, e.g. *R. solani*, or a strain of *Alternaria*, e.g. *A. brassicicola* (WO 94/03578), or variants thereof such as those described in WO 00/34450, or WO 01/92502.

Examples of proteases are the ALCALASE, ESPERASE, SAVINASE, NEUTRASE and DURAZYM proteases. Other proteases are derived from *Nocardiopsis*, *Aspergillus*, *Rhizopus*, *Bacillus alcalophilus*, *B. cereus*, *B. natto*, *B. vulgatus*, *B. mycoide*, and subtilisins from *Bacillus*, especially proteases from the species *Nocardiopsis sp.* and *Nocardiopsis dassonvillei* such as those disclosed in WO 88/03947, and mutants thereof, e.g. those disclosed in WO 91/00345 and EP 415296.

Examples of amylases are the BAN, AQUAZYM, TERMAMYL, and AQUAZYM Ultra amylases. An example of a lipase is the RESINASE A2X lipase. An example of a xylanase is the PULPZYME HC hemicellulase. Examples of endoglucanases are the NOVOZYM 613, 342, and 476 enzyme products.

Examples of mannanases are the *Trichoderma reesei* endo-beta-mannanases described in Ståhlbrand et al, J. Biotechnol. 29 (1993), 229-242.

Examples of steryl esterases. peroxidases, laccases, and cholesterol esterases are disclosed in the references mentioned in the background art section hereof. Further examples of oxidoreductases are the peroxidases and laccases disclosed in EP 730641; WO 01/98469; EP 719337; EP 765394; EP 767836; EP 763115; and EP 788547. In the present context, whenever an oxidoreductase enzyme is mentioned that requires or benefits from the presence of acceptors (e.g. oxygen or hydrogenperoxide), enhancers, mediators and/or activators, such compounds should be considered to be included. Examples of enhancers and mediators are disclosed in EP 705327; WO 98/56899; EP 677102; EP 781328; and EP 707637. If desired a distinction could be made by defining an oxidoreductase enzyme system (e.g. a laccase, or a peroxidase enzyme system) as the combination of the enzyme in question and its acceptor, and optionally also an enhancer and/or mediator for the enzyme in question.

These are particular embodiments of the present invention: Use of a fatty acid oxidizing enzyme for reducing the deposition of pitch in the paper making process. A process for reducing deposition of pitch in the paper making process, wherein the process comprises treating the pulp and/or process water with an enzyme preparation comprising a fatty acid oxidizing enzyme; preferably a process wherein the pulp is a mechanical pulp or a chemical pulp or a combination thereof; such as a chemical pulp. The process as described above, wherein the enzyme is classified in EC 1.13.11, preferably 1.13.11.12, preferably wherein the enzyme is derived from a strain of the genus *Magnaporthaceae, preferably M. salvinii* or the genus *Gaeumannomyces, preferably G. graminis*. The process described above, wherein the treatment is carried out by adding a substrate for the enzyme, preferably linoleic acid. The process described above, wherein the enzyme preparation comprises a lipolytic enzyme

and/or a further oxidoreductase. The process described above wherein the treatment is carried out at a temperature is in the range 20-90 °C, preferably 40-70 °C, and/or at a pH in the range 2-11, preferably 4-9.5, more preferably 6-9, and/or wherein treatment is carried out in 10 minutes to 3 hours, preferably 15 minutes to 1 hour; and/or wherein the enzyme is added in a concentration in the range of 0.0001-20 mg/g, preferably 0.0001-10 mg/g, more preferably 0.001-1 mg/g and most preferably 0.01-0.1 mg/g. In one embodiment of the above process the enzyme preparation is added in the storage chest or mixing chest before the paper machine.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

EXAMPLES

Example 1

Measurement of the activity of fatty acid oxidizing enzymes on linoleic acid

An "Oxi 3000 Oximeter" (WTW, Weilheim, Germany) with a TriOxmatic 300 oxygen electrode and a standard reaction volume of 4 ml was used.

10 mg linoleic acid (10 ml 60% linoleic acid) was dissolved in 1 ml ethanol, and 2 micro liter Tween 20 was added. From this stock substrate solution 50 micro liter was added into a reaction beaker containing 3.85 ml buffer solution (Britton-Robinson: 100 mM of Phosphoric-, Acetic- and Boric acid; pH adjusted with NaOH) with a small stir bar allowing the solution to be mixed well, and the oxygen electrode was inserted into the reaction beaker. 100 micro liter purified enzyme solution was added, viz. (a) lipoxygenase derived from *Magnaporthe salvinii* at a concentration of approx. 0.4 mg/ml; or (b) lipoxygenase derived from *Gaeumannomyces. graminis* at a concentration of approx. 0.76 mg/ml (which means approximately 0.02 mg/ml in the final reaction). These lipoxygenases were prepared as previously described. The temperature was 25°C. The concentration of dissolved oxygen (mg/l) is measured and plotted as a function of time (min.). The enzymatic activity is calculated as the slope of the linear part of the curve (mg/l/min.) after addition of the enzyme. The baseline was corrected by subtraction when relevant, meaning that if the curve showing oxygen concentration as a

function of time had a slope of above about 0.05 mg oxygen/ml/min before addition of the fatty acid oxidizing enzyme (i.e. the control), this value was subtracted from the sample slope value.

Table 1 below shows the results of the experiments.

Table 1

	Fatty Acid Oxidizing Enzyme	
pH	(a) LOX from *M. salvinii* mgO_2/ml/min	(b) LOX from *G. graminis* mgO_2/ml/min
2	0.0	0.0
4	0.4	0.1
5	0.7	0.4
6	1.1	0.4
7	1.0	0.4
8	0.7	0.5
9	0.8	0.4
10	0.7	0.4
11	0.6	0.2

Example 2

Fatty acid oxidizing enzymes

Four enzymes, viz. two laccases and two lipoxygenases were tested as described below. The laccase derived from *Polyporus pinsitus* had a MW by SDS-Page of 65 kDa, a pI by IEF of 3.5, and an optimum temperature at pH 5.5 of 60°C. The laccase derived from *Coprinus cinereus* had a MW by SDS-Page of 67-68 kDa, a pI by IEF of 3.5-3.8, and an optimum temperature at pH 7.5 of 65°C. The enzymes were prepared and purified as described in WO 96/00290 and US patent no. 6,008,029. The two lipoxygenases were derived from *Magnaporthe salvinii* and *Gaeumannomyces graminis*, and they were prepared as described previously.

The enzyme dosage was adjusted to ensure maximum absorbancy increase per minute at 234 nm / 530 nm, viz. in the range of 0.1 - 0.25 absorbancy units pr. min.

Substrate solution: 11.65 mg linoleic acid (60% Sigma), as well as 12.5 ml 0.56 mM Syringaldazine (Sigma) in ethanol was mixed with deionized water to a total volume of 25 ml.

50 microliter of the enzyme preparation to be tested was transferred to a quartz cuvette containing 900 microliter phosphate buffer (50 mM, pH 7.0) and 50 microliter of the substrate solution The cuvette was placed in a spectrofotometer, thermostated at 23°C, and the absorbancies at 234 nm and 530 nm were measured as a function of time. The absorbancy at 530 nm is indicative of degradation of syringaldazine, whereas the absorbancy at 234 nm is

indicative of degradation of linoleic acid. The absorbancy increase as a function of time is calculated on the basis of minutes 2 to 4 of the reaction time, i.e. $d(A_{234})/dt$, as well as $d(A_{530})/dt$.

The results are shown in Table 2 below. Of these four enzymes, only the two lipoxygenases qualify as a fatty acid oxidizing enzyme as defined herein. This is because RRD = Reaction Rate Difference = $(dA_{234}/dt - dA_{530}/dt)$ is above zero only for these two enzymes.

Table 2

Enzyme	dA_{530}/dt (units/min)	dA_{234}/dt (units/min)	$dA_{234}/dt - dA_{530}/dt$ (units/min)
Polyporus pinsitus laccase	0.20	0.002*	-0.20
Magnaporthe salvinii lipoxygenase	0.0001*	0.13	0.13
Coprinus cinereus laccase	0.17	-0.001*	-0.17
Gaeumannomyces graminis lipoxygenase	-0.03*	0.21	0.21

* this is equivalent to zero activity (analytical inaccuracy)

Example 3

Reduction of pitch with a fatty acid oxidizing enzyme

A model pitch is prepared as follows:

50 % Linoleic Acid 60% (Sigma L-1626).

20 % Abietic Acid A9424).

20 % Oleic Acid (Merck 471).

5 % Cholesterol-Linoleate (Sigma C-0289).

5 % Olive Oil (Sigma O-1500).

Mixed for 30 minutes at 65°C. Stored in refrigerator for no longer than 30 days.

Preparation of 0.1% pitch suspension:

50 mg model pitch

1 ml ethanol.

1 ml 0.1 M NaOH.

48 ml buffer (50 mM borate pH 9.0) Mixed for 10 minutes at room temperature.

Circular paper pieces (diameter = 5.5-6 mm; Multicopy 80 g/m^2) are transferred to the wells of two 96-well microtiterplates (ID 269620 from NUNC) designated A and B. Two other similar microtiterplates C and D are also used, but without paper pieces. 100 microliter of the 0.1% pitch suspension is added into each of the wells of each of these four microtiterplates. A lipoxygenase derived from *Magnaporthe salvinii* as described previously is used as the fatty acid oxidizing enzyme, and it is added to the wells of microtiterplates A and C to obtain an in-well-concentration of 10 ppm. A similar amount of buffer (50 mM borate pH 9.0) is added to the

wells of microtiterplates B and D. The microtiterplates are then incubated during shaking (600 rpm) for 30 minutes. After 30 minutes, 20 microliter of the enzyme-treated pitch suspension is transferred onto a second set of microtiterplates corresponding to microtiterplates A-D (Corning Inc. Costar UV plate 96 well No. 3635) each containing 200 microliter ethanol per well (solubilizing the pitch components). Abietic acid, a major component of the pitch, absorps strongly at about 255nm. Accordingly, A_{255} is indicative of the amount of pitch remaining in the suspension. A_{255} is determined as the average of 8 identical experiments, and the amount of pitch adsorbed onto the paper is estimated based on the variation in A_{255} measured in the pitch suspensions obtained after incubation with and without paper present (after 11x dilution in ethanol).

The results are shown in a table like the below Table 3. The basic (blind) adsorption of pitch onto the paper in the absence of a fatty acid oxidizing enzyme may be calculated as the ratio D/B. The effect of the enzyme (the sample) as regards the adsorption of pitch to the paper may be calculated as the ratio C/A. One way of showing hat the enzyme has caused a reduction in the deposition of pitch is if (C/A – D/B) is below zero. Alternatively, the enzyme effect may be calculated as ((C-A)-(D-B)), and if this value is below zero, this would be another way of showing the effect of the enzyme on the deposition of pitch. Other solid materials than paper may also be tested, e.g. metal, and textile (Style 400 cotton). The above ways of showing reduction in pitch deposition are applicable by analogy as regards deposition on the other solid materials.

Of course, the assay-pH (i.e. buffer), and the assay-temperature is selected paying regard to the characteristics of the fatty acid oxidizing enzyme in question, e.g. an assay pH of around 4, 5, 6, 7, 8, 9, 10, or 11; and an assay-temperature of around 10, 15, 20, 25, 30, 37, 40, 50, 60, 70, 80, 90 or 95°C.

Table 3

A_{255}	With *M. salvinii* lipoxygenase	Without *M. salvinii* lipoxygenase
With paper	A	B
Without paper	C	D

Example 4

Bleaching paper with a fatty acid oxidizing enzyme

Unbleached Kraft Pulp derived from *Eucalyptus grandis* was used. The pulp was repulped at 4% consistency in a pulper manufactured by Loretzen and Wettre. Repulping was done in buffer (Britton-Robinson) at pH=9.0.

Britton Robinson buffer:

100 mM Phosphoric acid (85%) 6.28 ml
100 mM Acetic acid (100%) 5.72 ml
100 mM Boric acid 6.18 g
Dem. water up to 1000 ml

The pH was adjusted to 9.0 by addition of sodium hydroxide.

After repulping the pulp slurry was diluted to 1% consistency by addition of buffer and pH was readjusted to pH = 9.0.

Treatments with a fatty acid oxidizing enzyme were carried out in beakers containing 3 g dry pulp i.e. 300 ml pulp slurry. The treatments were carried out at 25°C in a water bath with agitation by magnetic stir bars, 500 rpm. 333 ppm linoleic acid was added to all beakers. The fatty acid oxidizing enzyme used was a purified lipoxygenase derived from *Gaeumannomyces graminis* prepared as described previously. The amount of enzyme used appears from Tables 4 and 5 below. The enzyme treatment was carried out for 2 hours. Two beakers were run for each condition.

After two hours the enzyme reaction was stopped by addition of 5 ml (fixed amount) of NaOH (27.65% solution), this raises pH to > 12, and deactivates the enzyme.

The content of the beaker was transferred quantitatively to a 1000 ml beaker using 700 ml deionised water. This pulp suspension was poured onto a Büchner funnel (15 cm diameter) with a filter paper. A paper sheet was formed by sucking the water out. The paper sheet was removed from the funnel and separated from the filter paper. The sheet was pressed in a sheet press manufactured by Lorentzen and Wettre. The sheet was pressed in a sandwich of metal plate, 2 blotting papers, 2 filter papers, the sheet, 2 filter papers, 2 blotting papers, metal plate at 0,4 MPa for 5.5 min. Wet papers were replaced by dry ones and the pressing repeated at 0.4 MPa for 2 min. The sheets were air dried overnight.

The brightness of the sheets was measured using a Macbeth Color-Eye 7000 reflectometer. The brightness was recorded at 600nm. 4 measurements were done at each sheet. The results obtained are shown in Table 4 below.

The Kappa Number, which describes the degree of delignification of a pulp, was also determined for each sheet using the method described in Tappi Test Methods T236 (Tappi Press). The amounts used for each determination was ¼ of that described in the standard method. The dry matter content of the sheets was determined to calculate the Kappa no. The results obtained are shown in Table 5 below.

Table 4

LOX [mg/l]	Brightness Reflection at 600 nm Sheet 1 Average of 4 determinations	Brightness Reflection at 600 nm Sheet 2 Average of 4 Determinations	Brightness Reflection at 600 nm Average
0	46.93	46.60	46.77
0.1	49.69	50.23	49.96
1.3	48.51	47.15	47.83
3.2	49.16	50.12	49.64
6.3	48.56	52.56	50.56

Table 5

LOX [mg/l]	Kappa No. Sheet 1 Average of 3 determinations	Kappa No. Sheet 2 Average of 3 determinations	Kappa No. Average
0	18.44	18.43	18.44
0.1	14.80	14.68	14.74
1.3	14.94	14.93	14.94
3.2	14.65	14.42	14.54
6.3	14.83	14.40	14.61

Example 5

De-inking old newsprint with a fatty acid oxidizing enzyme

200 g shredded old newsprint was placed in a Hobart Mixer together with 1500 ml of water. The water bath temperature was set at 45°C. Mixing occurred at low speed for about 0.5 - 1 minutes. Then 3.6 kg/ton (7 lb/ton) of surfactant and 2 mg (10 mg/ton pulp) of a lipoxygenase derived from *Magnaporthe salvinii* prepared as described previously was added to the mixer, following which 500 ml of water was added to Hobart and mixed well. The mixer was run on low speed for 30 minutes. The pulper temperature was set at 45°C, and the pH at 7.

Half of the pulp was transferred from the mixer to a container and diluted to 10 l. Stirring took place for 2 minutes.

Feed pads: 30 ml of pulp was measured from the mixer and diluted to 300 ml with water and mixed well. The pulp was filtered through a Wattman#40 filter paper under vacuum. The pad was dried at 90°C (195°F) for 10 minutes.

Regular washing of pads: 900 ml of pulp slurry was measured from the container, poured slowly onto an 80 mesh sieve and shaken slowly until all free water drained. All pulp was removed and put into a 1000 ml beaker, which was filled with water up to the 900 ml line. The pulp was slowly stirred. 300 ml of the pulp slurry was measured and filtered through a Wattman#40 filter paper under vacuum. The pad was dried at 90°C (195°F) for 10 minutes.

Hyper-washing of pads: 900 ml of pulp slurry was measured from the container, poured slowly onto an 80 mesh sieve and shaken slowly until all free water drained. The pulp was rinsed with faucet water for 3 minutes, removed and put into a 1000 ml beaker, which was filled with water up to the 900 ml line. The pulp was slowly stirred. 300 ml of the pulp slurry was measured and filtered through a Wattman #40 filter paper to make a filter pad. The pads were dried on a speed dryer at 90°C (195°F) for 10 minutes.

The brightness of the pads was determined by a Macbeth color eye using a Tappi standard method (T452).

Comparative experiments were conducted as described above with two commercial enzymes, viz. the lipase RESINASE A 2X, and the cellulase DENIMAX L, both commercially available from Novozymes A/S, Krogshoejvej 36, DK-2880 Bagsvaerd, Denmark. These enzyme preparations were used in an amount of 0.51 kg per ton pulp (1 lb/t).

The results are shown in Table 6 below.

Table 6

Enzyme	Brightness		
	Feed Pads	Washed Pads	Hyper-washed Pads
Control (no enzyme)	38.5	41.7	45.9
Fatty acid oxidizing enzyme	38.1	44.0	48.2
RESINASE A 2X (lipase)	40.6	42.9	45.9
DENIMAX L (cellulase)	37.6	39.0	44.2

CLAIMS

1. Use of a fatty acid oxidizing enzyme in the manufacture of a paper material.

2. The use according to claim 1 for reducing the deposition of pitch.

3. The use according to any one of claims 1-2 for bleaching.

4. In a process for manufacturing a paper material from a papermaking pulp that comprises pulp from recycled, printed paper material, the use according to any one of claims 1-3 for de-inking.

5. The use according to any one of claims 1-4, wherein a chemical pulp forms part of the pulp being used for the manufacture of the paper material.

6. The use of a fatty acid oxidizing enzyme according to any one of claims 1-5 together with a substrate for the enzyme.

7. The use according to any one of claims 1-6 of a fatty acid oxidizing enzyme together with an additional enzyme having protease, lipase, xylanase, cutinase, oxidoreductase, cellulase, endoglucanase, amylase, mannanase, steryl esterase, and/or cholesterol esterase activity.

8. The use according to claim 7, wherein the additional oxidoreductase enzyme has laccase, and/or peroxidase activity.

9. The use according to any one of claims 7-8, wherein the additional enzyme has lipase activity.

10. A process for manufacturing a paper material, which process comprises the step of treating a papermaking pulp and/or process water with a fatty acid oxidizing enzyme.

11. The process according to claim 10, further comprising the steps of forming and drying the enzyme-treated pulp.

12. The process according to any one of claims 10-11, in which the enzyme-treatment results in

a) reduced deposition of pitch;

b) bleaching of the resulting paper material.

13. The process according to any one of claims 10-12, in which a chemical pulp forms part of the pulp being used for the manufacture of the paper material.

14. The process according to any one of claim 10-13, wherein the papermaking pulp comprises pulp from recycled printed paper materials, and wherein the enzyme-treatment results in a bleaching of the resulting paper material which is at least partly due to a de-inking effect of the enzyme.

15. The process according to any one of claims 10-14, wherein a substrate for the enzyme is added before or during the enzyme-treatment step.

16. The process according to any one of claims 10-15, further comprising a treatment of the papermaking pulp and/or process water with an additional enzyme having lipase, cutinase, oxidoreductase, cellulase, amylase, mannanase, steryl esterase, and/or cholesterol esterase activity.

17. The process according to claim 16, wherein the additional oxidoreductase enzyme has laccase, and/or peroxidase activity.

18. The process according to any one of claims 16-17, wherein the additional enzyme has lipase activity.

19. The process according to any one of claims 16-18, wherein the treatment with the additional enzyme occurs before, concomitantly with, and/or after the treatment with a fatty acid oxidizing enzyme.

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK 02/00697

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 D21C9/08 D21C9/10 D21C5/02 D21H21/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 D21C D21H D21F C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, PAPERCHEM

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO 00 53843 A (MUSTRANTA ANNIKKA ;VALTION TEKNILLINEN (FI); SPETZ PETER (FI); BUC) 14 September 2000 (2000-09-14) the whole document ---	1-19
A	WO 92 09741 A (ENSO GUTZEIT OY) 11 June 1992 (1992-06-11) the whole document ---	1-19
A	WO 92 13130 A (NOVONORDISK AS ;JUJO PAPER CO LTD (JP)) 6 August 1992 (1992-08-06) the whole document ---	1-19

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
20 January 2003	31 01 03

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/DK 02/00697

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